Diethylstilbestrol (DES)-Stimulated Hormonal Toxicity is Mediated by ERα Alteration of Target Gene Methylation Patterns and Epigenetic Modifiers (*DNMT3A*, *MBD2*, and *HDAC2*) in the Mouse Seminal Vesicle

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Abstract

Background: Diethylstilbestrol (DES) is a synthetic estrogen that is associated with adverse effects on reproductive organs. DES-induced toxicity of the mouse seminal vesicle (SV) is mediated by ERα with altered expression of seminal vesicle secretory protein IV (*Svs4*) and lactoferrin (*Ltf*) genes.

Objectives: We examined a role for nuclear receptor activity in association with DNA methylation and altered gene expression.

Methods: We used the neonatal DES exposure mouse model to examine DNA methylation patterns via bisulfite conversion sequencing in WT and αERKO SVs.

Results: DNA methylation status at 4 specific CpGs (-160, -237, -306 and -367) in the *Svs4* gene promoter changes during mouse development from methylated to un-methylated, and DES prevents this change at 10-weeks of age in WT SV. DES alters the methylation status from methylated to un-methylated at 2 specific CpGs (-449 and -459) of the *Ltf* gene promoter. Alterations in DNA methylation of *Svs4* and *Ltf* were not observed in αERKO SV, suggesting that changes of methylation status at these CpGs are ERα dependent. The methylation status associates with the level of gene expression. In addition, gene expression of three epigenetic modifiers, including *DNMT3A, MBD2*, and *HDAC2* increased after DES exposure in WT SV.

Conclusion: DES-induced hormonal toxicity results from altered gene expression of *Svs4* and *Ltf* associated with changes in DNA methylation that are mediated by ERα. Alterations in gene expression of *DNMT3A, MBD2* and *HDAC2* after DES exposure may be involved in mediating the changes in methylation status in the SVs of male mice.

Introduction

Endocrine-disrupting chemicals (EDCs) are substances in the environment, food sources, and manufactured products that can interfere with the normal functioning of our body's endocrine system (Diamanti-Kandarakis et al. 2009). EDCs include synthetized or natural hormones, pharmaceuticals, pesticides, and/or plasticizers that influence activity of estrogen receptors (ERs) (Henley et al. 2009). Diethylstilbestrol (DES) was the first orally active synthetic estrogen that was used to treat pregnant women with the intent of facilitating placental steroidogenesis and reducing the risk of spontaneous abortion or pre-term parturition (Marselos and Tomatis 1992a, b). In 1971, clinical studies found that DES causes a rare vaginal tumor in young women exposed to this drug *in utero* (Greenwald et al. 1971; Herbst et al. 1971). The US FDA blocked the use of DES for pregnancy support almost immediately thereafter (Herbst 2000).

A mouse model of neonatal DES exposure was widely used to study the possible effects of DES on the reproductive organs (McLachlan 1977; McLachlan and Dixon 1977). Additionally, the model system has been used to help elucidate the mechanism (s) of hormonal carcinogenics (McClain et al. 2001). Studies indicate that female mice treated neonatally with DES develop a high incidence of uterine adenocarcinoma (Newbold et al. 1990). Similarly treated male mice develop testicular cancer and abnormalities of the prostate and seminal vesicles (SVs) (McLachlan 1977; McLachlan and Dixon 1977). Prins et al. showed that neonatal estrogen (E2) exposure induced lobe-specific alterations in the adult rat prostate, including a permanent decrease in androgen receptor (AR) levels (Prins 1992; Prins et al. 1993). Studies using the neonatal DES exposure model report an abnormal morphology of the penis in male rats associated with changes in the protein levels of ERα, but not AR (Prins and Bremner 2004).

Neonatal DES exposure decrease the protein level of ERα in the anterior prostate significantly, but increases its level in the SV of male mice (Turner et al. 1989).

The biological effects of E2 and some EDCs are mediated through the ERs (ERα and ERβ), which are members of a large super-family of nuclear receptors. These receptors act as ligand-inducible transcription factors (Hall and McDonnell 2005). The classical mechanism of ER action is characterized by ER directly binding to estrogen response elements (EREs) of target genes. The non-classical mechanism is the "tethered" mechanism where ERs regulate the expression of a large number of E2-responsive genes through interaction with other transcription factors such as *c-Jun*, *c-Fos* or *Stat5* (Bjornstrom and Sjoberg 2005). EDCs regulate many target genes through ER similar to the regulation by E2 (Moggs et al. 2004).

Seminal vesicle secretory protein IV (SVS IV) is an androgen-dependent protein (Chen et al. 1987). The expression of the *Svs4* gene is dependent on the presence of testosterone in the rat SV (Higgins et al. 1976; Higgins et al. 1981). Lactoferrin (*Ltf*) is a female specific gene and serves as an appropriate marker of estrogenic action due to its high level of RNA and protein expression in E2 stimulated uteri compared to other tissues (Pentecost and Teng 1987). Prenatal DES exposure studies show the expression levels of the *Ltf* gene are now induced in the SV from DES-treated mice (Newbold et al. 1989).

Our research group has used the ER knockout (ERKO) mouse to study ER-dependent pathways involved in mediating the effects of neonatal DES exposure in the reproductive tract tissues of the mouse (Couse et al. 2001; Couse and Korach 1999). These results demonstrate that ERα plays a critical role in mediating the toxicological effects of neonatal DES exposure in female and male reproductive tracts (Couse et al. 2001; Couse and Korach 2004). In the prostate, we

4

found that E2 imprinting of the developing prostate gland was mediated through stromal ERα (Prins et al. 2001). DES exposure decreased SV weight in wild type (WT) mice at 4 months of age but not in αERKO mice (Couse and Korach 2004; Prins et al. 2001). Recently, we reported that DES-induced SV toxicity and feminization were primarily mediated through ERα in adult mice (Walker et al. 2012).

DNA methylation is a well-characterized epigenetic modification and is important for gene regulation, transcriptional silencing, development, and tumorigenesis (Esteller 2008; Feinberg and Tycko 2004; Jones and Baylin 2007; Wu and Zhang 2010). In mammalian cells, DNA methylation occurs at the 5' position of the cytosine ring within CpG dinucleotides via addition of a methyl group to create a 5'-methylcytosine (5mC). The methylation at 5mC is catalyzed by DNA methyltransferases (*DNMTs*), including *DNMT1*, *DNMT3A* and *DNMT3B* (Bestor 2000; Chen and Li 2004). The DNA methylation pattern is believed to be "read" by a conserved family protein, the methyl CpG binding domain (MBD) family (Jaenisch and Bird 2003; Wade 2001). MeCP2, MBD2 and MBD3 proteins belong to the MBD family and they represent an important class of chromosomal proteins, which associate with protein partners that play active roles in transcriptional repression and/or heterochromatin formation (Wade 2005). The second well-known epigenetic mechanism is histone modification and it is critical for regulating chromatin structure and function (Jenuwein and Allis 2001; Luger et al. 1997). Histone deacetylases (HDAC) 1 and 2 are highly conserved enzymes that help regulate chromatin structure as the core catalytic components of corepressor complexes (Brunmeir et al. 2009). To date, studies indicate that these epigenetic markers play an important role during development in transcriptional

programs. However, the correlation between DNA methylation and gene expression and the involvement of these epigenetic makers after EDC exposure are still poorly understood.

In this study, we used a neonatal DES exposure mouse model to examine the changes of DNA methylation patterns in the altered androgen dependent gene, *Svs4* and the estrogen dependent gene, *Ltf*, and their correlation of the methylation status with gene expression. Furthermore, we evaluated the role of ERα in the DNA methylation process and alterations in gene expression of epigenetic markers in the seminal vesicle of male mice.

Materials and Methods

Chemicals

Diethylstilbestrol (DES, CAS no. 56-53-1) was purchased from Sigma-Aldrich (St. Louis, MO).

Animals and neonatal DES treatment

All animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the National Institute of Environmental Health Science (NIEHS) Animal Care and Use Committee. Animals were treated humanely and with regard for alleviation of suffering. Mice were housed under constant environmental conditions (22 ± 1 °C; relative humidity, 40% to 60%; 12:12-h light: dark cycle). Mice received autoclaved feed (NIH31 pelleted chow, Zeigler Brothers, Gardners, PA) and reverse-osmosis deionized water ad libitum and were housed in polycarbonate caging with hardwood bedding (SaniChip, PJ Murphy Forest Products, Montville, NJ) with autoclaved environmental enrichment (Nestlets, Ancare, Bellmore, NY). For wild type (WT) mice, 8-12 week old pregnant C57Bl/6 females (total numbers: 30) were obtained from Charles River Laboratories (Wilmington, MA). ERα null mice (αERKO) were generated by breeding C57Bl/6 heterozygous (ERα+/-) animals as described previously (Couse et al. 2003). On the day of birth (considered 1 day of age), male pups from

6

multiple litters were pooled and randomly distributed among 8-12 week old CD-1 foster mothers with 8 pups per dam. For neonatal treatment, pups were treated each morning by subcutaneous injection with DES dissolved in corn oil at 2 µg/pup/day (0.02 cc) on days 1-5 (day of birth = 1) (DES group) or an equal volume of corn oil (vehicle group). Mice (WT and αERKO) were weaned and were genotyped at 21 days of age. After weaning, mice were housed 2-4 per cage based on prior treatment group (corn oil as vehicle or DES). The genotyping was performed by PCR on DNA extracted from tail biopsy using previously described methods (Couse et al. 2003). Mice were euthanized by CO_2 inhalation and immediately, seminal vesicle (SV) tissues were collected at week 3, 5, and 10 from WT vehicle- or DES-treated groups and only at week 10 from αERKO vehicle- or DES-treated groups (Figure 1). The SV tissues were snap frozen and kept at -80 °C until use.

RNA extraction and real-time PCR

Total RNA samples were extracted from frozen SV tissues of individual mice by using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using Superscript reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The mRNA levels of genes (*Svs4, Ltf, Pgr, Stat3, Stat5a, DNMT1, DNMT3A, DNMT3B, MeCP2, MBD2, MBD3, HDAC1* and *HDAC2*) were measured using SYBR green assays (Applied Biosystems). The sequences of primers used in real-time PCR are listed in Supplemental Material, Table S1. Cycle threshold (Ct) values were obtained using the ABI PRISM 7900 Sequence Detection System and analysis software (Applied Biosystems, Foster City, CA, USA). The experiments were repeated three times and results are presented as fold increase calculated relative to the vehicle (control) of WT SV ± SE at week 5.

7

Identification of potential ERE sequences and CpGs

The genomic sequence of the gene promoters (*Svs4*, *Ltf* and *Pgr*) was downloaded from the UCSC genome Browser (genome.ucsc.edu, build mm10). Putative ERE sequence with the position weight matrix (PWM) constructed from 48 experimentally identified EREs (15-bp in length) was scanned by using the GADEM software (Jin et al. 2004; Li 2009). CpGs were identified by using the software program EpiDesigner (http://www.epidesigner.com/).

DNA extraction and bisulfite conversion sequencing PCR

Genomic DNA (400-500 ng) was extracted from frozen SV tissues of individual mice using a Tissue Blood Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Bisulfite conversion sequencing PCR primers as shown in Supplemental Material, Table S2 were designed using the software program EpiDesigner (http://www.epidesigner.com/). Bisulfite conversion sequencing PCR was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. The PCR products were resolved on a 2% agarose gel and purified by using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

Cloning and sequencing bisulfite-conversed DNA

Purified PCR product from individual mice was subcloned into the pCR-TOPO-XL vector using TOPO XL PCR Cloning Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Six or more clones were picked and sequenced for each sample. The sequencing analysis of bisulfite-conversed DNA was performed using CpGviewer program (Carr et al. 2007). The data was presented from three individual mice.

Statistical analysis

One-way ANOVA with Dunnett's Multiple Comparison test (*, $p < 0.05$, **, $p < 0.01$, Figure 5) and Two-way ANOVA with Bonferroni post-test (***, $p < 0.001$, Figure 2) were performed using GraphPad Prism version 6.00 (San Diego, CA, USA).

Results

DES-altered levels of *Svs4* and *Ltf* gene expression are ERα dependent after neonatal DES exposure

To verify alteration of androgen/or estrogen-dependent genes after neonatal DES exposure in the SV tissues, we examined *Svs4*, a male specific gene, and *Ltf*, a female specific gene in WT and αERKO adult male mice. The *Svs4* gene is highly expressed in the WT SV. In SVs collected 10 weeks after neonatal DES exposure, the expression level of *Svs4* decreased greater than 90% compared to the control group (Figure 2A). The expression level of *Svs4* in the SV was much lower in the αERKO control group compared to the WT control group and there was no significant change in the αERKO DES groups (Figure 2A). In addition, at week 5 expression of the *Svs4* gene was also lower in both WT control and DES groups than in the WT control at week 10 SVs (data not shown).

We found minimal *Ltf* gene expression in both WT and αERKO vehicle SVs (Figure 2B). Interestingly, in 10-week old neonatal DES exposed SVs, high levels of *Ltf* gene expression was found in WT but not in the αERKO SVs. In addition, we used immunohistochemistry staining to detect the levels of lactoferrin (LF) protein and found strong staining by the LF antibody in the WT SV but not in the αERKO SV after neonatal DES exposure (Figure S1). These data confirm that ERα mediates DES-induced alterations of gene expression in the SV of adult male mice.

The methylation status of four specific CpGs in the *Svs4* gene promoter change during development from methylated to un-methylated while DES and ERα prevent this methylation change

Using data from UCSC Genome Browser, we found four CpGs (-160, -237, -306 and -367) located in the *Svs4* gene promoter close to the transcription start site (Figure 3A). To investigate whether DNA methylation correlates with *Svs4* transcription, we used bisulfite sequencing to examine the methylation status of four CpGs in the *Svs4* gene promoter in WT SVs at 3, 5 and 10 weeks of age. In the control group, we found over 70% of these CpGs were methylated in WT at 3 and 5 weeks of age, but only 18% of the same CpGs were methylated at week 10 (Figure 3B, top). However, in the DES-treated group, about 60% of the CpGs at week 3 or 5 and 84% at week 10 were methylated (Figure 3B, bottom). The maintenance of methylation at these CpGs is consistent with down-regulation of the *Svs4* gene in the DES group at week 10 (Figure 2A).

To examine the effects of ERα on the DNA methylation status of the *Svs4* gene promoter, we performed bisulfite conversion sequencing PCR with the 10-week old αERKO SV samples. In both control and DES αERKO groups, over 70% of these four CpGs were methylated (Figure 3C). These results suggest that the lack of ERα and neonatal DES treatment block the normal developmental alterations in the DNA methylation status of four specific CpGs (-160, -237, -306 and -367) of the *Svs4* gene promoter in the SVs of adult male mice. Also, alterations of DNA methylation correlate with gene expression.

The methylation status of two specific CpGs in the *Ltf* gene promoter changes from methylated to un-methylated after neonatal DES exposure

 We searched the *Ltf* gene promoter and found five CpGs (-449, -459, -470, -528 and -542) located close to a well-characterized ERE site (-324) (Li et al. 1997; Liu and Teng 1992) and the

transcription start site (Figure 4A). To investigate whether the alteration of DNA methylation directly regulates the *Ltf* gene transcription, we examined the methylation status of these five CpGs in the *Ltf* gene promoter in the WT SV collected at weeks 3, 5, and 10 after neonatal DES treatment. In the control group, over 90% of these CpGs were methylated and the methylation pattern did not change during development from weeks 3 to 10 (Figure 4B, top). In the neonatal DES treated SV, there were no changes in the methylation status at week 3 or 5, but at week 10, two specific CpGs (-449 and -459) changed from methylated to un-methylated (Figure 4B, bottom). Loss of methylation at these CpGs is consistent with the up-regulation of the *Ltf* gene expression in the DES group at week 10 (Figure 2B).

When examining the methylation status of the five CpGs in the *Ltf* gene promoter of αERKO SV at 10 weeks of age, we found no change in the methylation status of two specific CpGs (-449 and -459) after neonatal DES treatment (Figure 4C). These data suggest that DES alters the methylation status of two specific CpGs (-449 and -459) from methylated to predominantly un-methylated in the *Ltf* gene promoter and the absence of ERα block this change in adult male mice.

Differential effects on the expression levels of epigenetic modifiers, DNMT3A, MBD2 and HDAC2 following neonatal DES exposure

To examine whether the altered methylation patterns from neonatal DES exposure might be affected by the expression level of the epigenetic modifiers, we used real time-PCR to investigate the RNA levels of DNA methytransferases, including *DNMTs (DNMT1, DNMT3A and DNMT3B)* and *MBDs (MeCP2, MBD2* and *MBD3)*, and a group of histone modifiers

11

HDACs (*HDAC1* and *HDAC2)*. We examined the expression levels of these genes in WT SV collected at week 5 or 10 after neonatal DES exposure.

The expression level of the *DNMT3A* gene increased in the DES group significantly at week 5 compared to week 5 vehicle groups, but *DNMT3A* gene decreased in DES group at week 10 compared to week 10 vehicle group (Figure 5A). However, the *DNMT1* and *DNMT3B* gene expression did not change after neonatal DES exposure in either week (Figure 5A). The level of *MeCP2* gene expression was much higher in the vehicle group at week 5 but this level significantly decreased relative to vehicle after neonatal DES exposure (Figure 5B) at this time point. However, no significant changes were seen in MeCP2 gene expression when comparing the vehicle and the DES groups at week 10. *MBD2* expression was significantly elevated in the DES group relative to the vehicle group. In addition, DES did not have an effect on the expression of *MBD3* relative to vehicle at 5 or 10 week of age (Figure 3B).

Expression of the histone modification marker, *HDAC2* significantly increased after neonatal DES exposure only at week 5 relative to vehicle, but not at week 10. In contrast, expression of *HDAC1* did not change in the DES group at week 5 or 10 (Figure 3C). These findings suggest that alterations in gene expression of these epigenetic modifiers are correlated with changes in methylation status seen with neonatal DES treatment.

Discussion

Alterations of DNA methylation correlate with the expression levels of specific genes after neonatal DES exposure in the SV of adult male mice

Recently, there is an increased interest in the effect of EDCs on human health (Henley et al. 2009). The most classical EDC, DES is still used as a great tool to study the possible effects of

12

EDCs on reproductive organs. Our previous studies indicated alterations in male and female specific genes after neonatal DES exposure in adult male mice (Couse and Korach 2004; Walker et al. 2012). In the present study, we investigated changes of DNA methylation patterns of the *Svs4* (male specific) and *Ltf* (female specific) genes during development or after neonatal DES exposure in WT and αERKO SVs. One of the most significant findings of our study is that there is a correlation between DNA methylation patterns and the levels of the *Svs4* and the *Ltf* gene expression in an ERα dependent matter after neonatal DES exposure in adult male mice.

Evidence shows the decreased *Svs4* expression is not due to a significant change in the level of *AR* gene expression in the SVs of adult male mice after neonatal DES exposure (Turner et al. 1989; Walker et al. 2012). SVS IV protein is found in the SVs of mice and rats and is regulated by androgen (Chen et al. 1987). Data from UCSC Genome Browser was used to analyze the *Svs4* gene promoter. We found a putative Stat5a/5b binding site at upstream (-132/-146 bp) and a Sp1 site at downstream (+118/+128 bp) (Figure S2). We found a predicted ERE site surrounding the 2 kb of the transcription start site (+/-1 kb). However, the 4 bases of the palindrome differ from the 10 base consensuses ERE (GGTCAnnnTGACC) (Figure S2). The SV weight of the WT adult male mice significantly decreased after DES exposure but this change did not occur in αERKO mice (Couse and Korach 2004). These data suggest that this effect is ERα-dependent and might act through the non-classical (tethered) mechanism. In the SV tissues from rat, a methylation-sensitive restriction assay showed that seven potential methylation sites were largely methylated (Kandala et al. 1985). In this study, we found a normal developmental change in DNA methylation status at four specific CpGs (-160, -237, -306, and -367) of the *Svs4* gene promoter in WT SVs from week 3 to week 10. Furthermore, we conclude that DES exposure and the

absence of ERα both block the normal developmental de-methylation of the *Svs4* gene and these changes in DNA methylation correlate with the gene expression from this study. This is the first report addressing the correlation between DNA methylation and expression of the *Svs4* gene in a mouse model and a role for ERα in this process. Our results help to explain the relationships of epigenetic mechanisms and gene regulation.

The *Ltf* gene, a well-known female specific ER target gene is upregulated by E2 in the female reproductive tract (Teng 2002). In female mice, the early appearance of LF protein expression suggests that it may play an important role on the hormonal regulation of growth and differentiation of developing uterine tissues (Newbold et al. 1997). In male mice, there is normally no *Ltf* gene expression or potential role for this gene in SV tissues; however, *Ltf* is highly expressed after neonatal/prenatal DES exposure (Couse and Korach 2004; Newbold et al. 1989). E2 increases the *Ltf* expression through a well-characterized ERE (-324) located upstream from the *Ltf* gene promoter transcription start site (Liu and Teng 1992; Liu et al. 1993). Using ChIP-qPCR, we confirmed that ERα is bound to this ERE (-324) site in the *Ltf* gene promoter in 10-week old WT SVs with or without neonatal DES exposure. However, DES enhances the enrichment of ERα binding after DES exposure (data not shown). In addition, we found three predicted EREs and a Sp1 site in the region 1 kb upstream of the *Ltf* gene promoter (Figure S3). Importantly, we found two specific CpGs (-449 and -459) upstream of the *Ltf* gene promoter that are altered in DNA methylation status from methylated to un-methylated after neonatal DES exposure. Methylation analysis in the CD-1 mouse uterus showed that prenatal DES exposure only altered one CpG (-459; the number in the reference, Li et al. 1997, was -464) from methylated to un-methylated, suggesting the effects of DES on DNA methylation are tissue and

/or stain-specific. Furthermore, DNA methylation status changed from methylated to un-methylated during development at three specific CpGs (-470, -528, and -542; the numbers in the reference were -475, -533, and -547) in the *Ltf* gene promoter in the 3-week old CD-1 mouse uterus (Li et al. 1997). Our data indicated that there were no developmental changes at these three CpGs (-470, -528 and -542) at week 3 in SV tissues from C57Bl/6 male mice. These findings suggest that there are sex and/or strain differences in the DNA methylation patterns of the *Ltf* gene in the mouse reproductive organs.

Next, we examined the gene expression level of the progesterone receptor (*Pgr*), a well-known ER target gene. The gene profile showed that there were differences in baseline expression of the *Pgr* gene due to the lack of ERα but there were no significant changes of *Pgr* expression in the SVs of WT and αERKO mice after neonatal DES treatment (Figure S4A). Using the software program EpiDesigner, we found a high CpG content, with twenty CpGs located in the introns of the *Pgr* gene between +661 and +886. When examining the DNA methylation patterns, we found that almost 100% of these CpGs were unmethylated in both control and DES groups in the SVs of 10-week old mice (Figure S4B), suggesting that the DNA methylation patterns of the *Pgr* gene correlate with its gene expression. There might be other CpGs of the *Pgr* gene involved in reduction of this gene expression in the αERKO SV when compared with WT samples; however, in the αERKO it is most likely that the lack of ERα and hormone responsiveness in the SV is the reason for lower expression of *Pgr*. The growth hormone signaling activated transcription factors, *Stat3* and *Stat5a* regulate estrogen signaling (Yamamoto et al. 2000; Hewitt et al, 2010). In this study, we found that the expression levels of the *Stat3* and *Stat5a* genes increased after neonatal DES exposure significantly at week 5, but not at week 10 in the SVs (Figure S5).

15

Furthermore, when we focused our efforts on ERα, we reconfirmed that there were no gene expression changes in the *AR* after neonatal DES exposure relative to vehicle levels (data not shown); demonstrating to us that alteration from DES exposure in the male was through another nuclear receptor. In addition, the level of serum testosterone is decreased in the adult αERKO mice compared with WT and this may regulate the levels of *Svs4* and *Ltf* gene expression (Walker et al, 2012).

Neonatal DES exposure alters the expression levels of epigenetic modifiers such as *DNMT3A*, *MBD2*, and *HDAC2* in the SV of male mice

There are two main epigenetic mechanisms/modifications, including DNA methylation and histone modification (Gabory et al. 2011). The enzymatic machinery for DNA methylation is composed mainly of three DNA methyltransferases (*DNMTs*), including *DNMT1*, *DNMT3A* and *DNMT3B* (Bestor 1988). In recent years, the level of these enzymes have been measured in reproductive organs as a group of DNA methylation markers after exposure to EDCs, such as DES and bisphenol A (BPA) (Bromer et al. 2010; Sato et al. 2006, 2009). Following neonatal DES treatment in mice, the uterus and epididymis have altered expression levels of *DNMT1*, *DNMT3A* and *DNMT3B* gene expression changes dynamically (Sato et al. 2006, 2009). Our present study showed that only *DNMT3A* expression increased in the SV of the adult mice that were neonatal exposed to DES, suggesting its involvement in epigenetic programming at different periods during development. Interestingly, expression of *DNMT3A* and *DNMT3B* was much lower in the DES group than in the vehicle group at week 10. These data demonstrate that these epigenetic modifiers change dynamically during DES exposure. The MBD family proteins such as *MeCP2*, *MBD2*, and *MBD3* play an important role in transcriptional repression (Bird et al. 1999; Hendrich and Tweedie 2003; Wade 2001). We found a significant increase of *MBD2*

16

expression in neonatal DES-treated adult mice (week 10) in this study. Our finding agrees with the report showing that *MBD2* expression increased after neonatal E2/BPA exposure in the rat prostate gland (Tang et al. 2012). The histone modification markers, *HDACs*, are evolutionarily conserved enzymes that remove acetyl modifications from histones and play a central role in epigenetic gene silencing (Hayakawa and Nakayama 2011). *HDAC1* controls embryonic stem cell differentiation, but no *HDAC2* effect was seen (Dovey et al. 2010). After neonatal DES exposure, we found that *HDAC2* expression was increased significantly in 5-week old SVs. These data demonstrate that the involvement of these histone modifiers in epigenetic programming could be cell and/tissue-type specific. The observed changes in these proteins suggest that the effects of DES on DNA methylation of target genes may be more widespread and a global analysis needs to be performed in future studies.

Conclusions

In this study, we found an association between DNA methylation and gene expression for the *Svs4* and the *Ltf* genes. A working model of this study is shown in Figure S6. Four specific CpGs (-160, -237, -306, and -367) in the *Svs4* gene changed from un-methylated to methylated during development and neonatal DES exposure prevented the developmental methylation change of these CpGs. Normal methylation changes in the *Svs4* gene were not seen in the αERKO linking an active role for ERα in the methylation changes. DES alters the DNA methylation status from methylated to un-methylated at two specific CpGs (-449 and -459) in the *Ltf* gene promoter. In addition, DES exposure regulates the expression levels of epigenetic modifiers, *DNMT3A*, *MBD2*, and *HDAC2*, significantly. Taken together, these results are consistent with the hypothesis that DES-induced toxicity is mediated by ERα alteration of target gene methylation

patterns and through changes in gene expression of three epigenetic modifiers after neonatal

DES exposure in the SV of adult male mice.

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Figure Legends

Figure 1. Experimental protocol for neonatal DES treatment and tissue collection in this study.

Figure 2. Neonatal DES exposure regulates gene expression in WT SV of adult mice. Total RNA samples were extracted from SV tissues of three individual 10-week old WT or αERKO mice after neonatal exposure to vehicle or DES. mRNA levels were quantified by real time-PCR. Data shown represent mean fold change (± SE) relative to the WT SV vehicle samples at week 5, ***, $p < 0.001$ by two-way ANOVA with Bonferroni post-test. (A) *Svs4* gene expression. (B) *Ltf* gene expression.

Figure 3. The methylation status of *Svs4* changes during development and after neonatal DES exposure. (A) The diagram depicts of four CpGs in the mouse *Svs4* gene. (B) DNA methylation status of the *Svs4* gene during development in WT SV. Genomic DNA was extracted from week 3, 5 or 10 WT SV tissues of individual mice. The region containing the four CpGs was amplified by PCR from bisulfite-treated genomic DNA, and then PCR product was subcloned into the pCR-TOPO-XL vector. The sequencing analysis of bisulfite-conversed DNA was performed using CpGviewer program. Each line of circles indicates an individual clone sequenced in the analysis after bisulfite treatment and PCR. Open circles indicate un-methylated CpGs. Black circles indicate methylated CpGs. Yellow circles indicate undetectable CpGs (unknown). Data shown represent the results from three individual mice. The percentages of methylated (Me)/un-methylated (un-me) CpGs represent the results from all four CpGs. (C) DES alters DNA methylation of the *Svs4* gene in adult male mice (week 10). Genomic DNA was extracted from 10-week old WT or αERKO SV tissues after neonatal exposure to control or DES. Bisulfite sequencing analysis was performed as described above.

Figure 4. The methylation status of *Ltf* changes during development and neonatal DES exposure. (A) The diagram depicts of five CpGs in the mouse *Ltf* gene promoter. (B) DNA methylation status of the *Ltf* gene during development in WT SV. Genomic DNA was extracted from week 3, 5 or 10 WT SV tissues of individual mice. Bisulfite sequencing was performed as described in Figure 3. Each line of circles indicates an individual clone sequenced in the analysis after bisulfite treatment and PCR. Open circles indicate un-methylated CpGs. black circles indicate methylated CpGs. Yellow circles indicate undetectable CpGs (unknown). Data shown represent the results from three individual mice. The percentages of methylated (Me)/un-methylated (un-me) CpGs represent the results from the two CpGs (-449 and -459). (C) DES alters DNA methylation of the *Ltf* gene in adult male mice (week 10). Genomic DNA was extracted from 10-week old WT or αERKO SV tissues after neonatal exposure to control or DES. Bisulfite sequencing analysis was performed as described above.

Figure 5. Changes of epigenetic markers, *DNMTs*, *MBDs* and *HDACs* after neonatal DES exposure in WT SV. Total RNA samples were extracted from SV tissues of three individual 5- or 10-week old WT or αERKO mice after neonatal exposure to vehicle or DES. The expression levels of *DNMTs* (A), *MBDs* (B) and *HDACs* (C) were quantified by real time-PCR. Data shown represent mean fold change (± SE) relative to the WT SV vehicle samples at week 5, *, $p < 0.05$, **, $p < 0.01$ by One-way ANOVA with Dunnett's Multiple Comparison test.
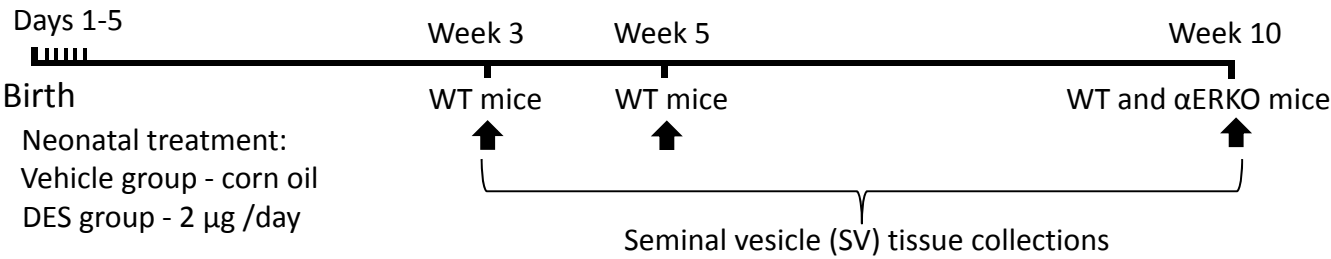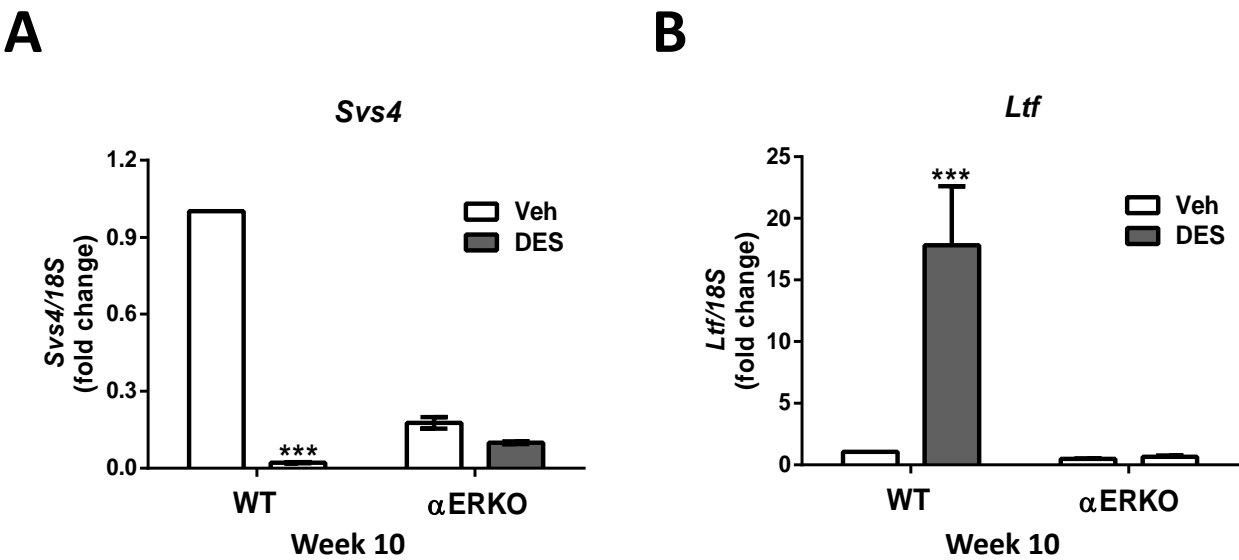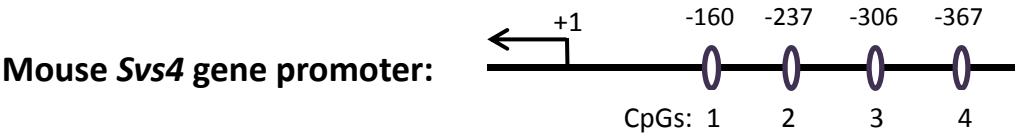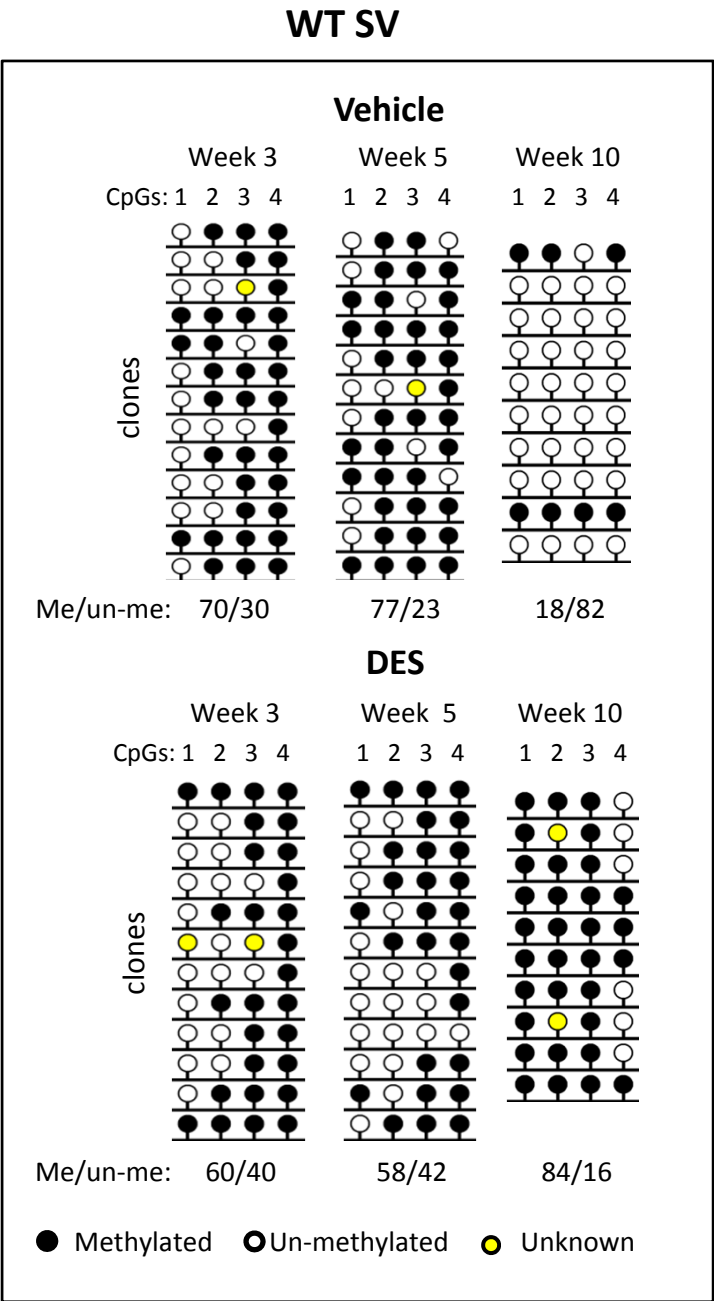
Figure 1

Days 1-5

Week 3   Week 5   Week 10

Birth

WT mice   WT mice   WT and αERKO mice

Neonatal treatment:
Vehicle group - corn oil
DES group - 2 µg /day

Seminal vesicle (SV) tissue collections

Figure 2

Figure 3

A

Mouse *Svs4* gene promoter:

B WT SV

C αERKO SV

Figure 4

Figure 5